Theor Appl Genet (1987) 73:489–495

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Starch characteristics in cultures of normal and mutant maize endosperm*

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Received May 15, 1986; Accepted October 10, 1986
Communicated by P. L. Pfahler

Summary. Vigorously growing suspension cultures of 'normal', *amylose-extender* (*ae*) and *waxy* (*wx*) maize endosperm were established from near isogenic lines of maize inbred A636. The recovery of the ability to produce vigorous cultures of *ae* and *wx* endosperm by backcrossing demonstrate the genetic control of endosperm growth in vitro. Phenotypic expression of the endosperm mutants in culture was studied by examining the properties of starch accumulated in endosperm cultures and starch from developing and mature kernels of the same genotype. After 9 months in culture, the amylose contents of the starch in normal callus tissue and normal endosperm tissue were not significantly different, 28.2% and 31.7%, respectively. Starch granules from normal cultures and endosperm stained blue-black with iodine and were round to polygonal in shape. The starches of *wx* endosperm and callus cultures contained no amylose, and *wx* starch granules stained brown-orange with iodine. Although, *wx* starch granules were primarily round, a few granules with "jagged edges" were observed in starch samples isolated from cultures and kernels. The percent amylose in starch from *ae* callus was significantly lower than the amylose content of starch from *ae* endosperm tissue, 39.9% and 67.7%, respectively. Starch granules from *ae* endosperm and cultures were smaller than normal and *wx* starch granules. Irregular starch granules which are typical of *ae* endosperm were present in *ae* callus tissue, but were less frequently observed. We conclude that specific endosperm mutant phenotypes are expressed in vitro.

Key words: Starch – Endosperm – Suspension cultures – Maize – *Zea*

Introduction

Methods for culturing maize endosperm tissue were first reported by LaRue (1949). In this study, a *sugary* endosperm line was used. Subsequently, several researchers attempted to culture various types of maize endosperm with limited success (Straus and LaRue 1954; Tamaoki and Ullstrup 1958; Tabata and Motoyoshi 1965; Graebe and Novelli 1966). Shannon and Batey (1973) were first to successfully culture starchy endosperm. Out of 23 starchy inbreds and hybrids tested, they found 2 inbred lines, A363 and R168, which produced explants that exhibited satisfactory callus formation. Shannon and Batey (1973) concluded that the ability to produce vigorous growing explants was under genetic control. Cultures of A636 were maintained for several years and used in studies of culture growth and starch biosynthesis (Boyer and Shannon 1974; Chu and Shannon 1975; Shannon and Liu 1977). Methods for rapid growth of maize endosperm cultures were tested (Shannon and Liu 1977) and Linsmair and Skoog (1965) salts with 0.4 mg/l thiamine and 2 g/l asparagine were found to give the best growth. Endosperm in liquid suspension cultures could be stimulated to accumulate starch (Chu and Shannon 1975). The highest starch accumulation was observed when cultures were supplemented with 6 g of sucrose 9 days after subculture.

* Supported in part by the United States Department of Agriculture Competitive Grant 85-CRCR-1-1740. Contribution No. 94, Department of Horticulture. The Pennsylvania State University. Authorized for publication as paper No. 7373 in the journal series of the Pennsylvania Agricultural Experiment Station

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Using the methods for culture initiation developed by Shannon and coworkers, Shimamoto et al. (1983) conducted studies on the expression of the zein endosperm storage protein fraction in maize endosperm cultures. They showed the presence of zein in 6-month-old cultures and also during the following 15 months. The endosperm cultures they established were of 2 types: yellow tissue which contained mainly protein bodies and white tissue which contained none or few protein bodies and some large starch granules. The protein bodies present in their cultures were equivalent in size to those found in 20-day-old kernels.

Although endosperm cultures have been used for starch biosynthesis and storage protein studies, the properties of cultures of the endosperm mutants of maize have not been examined. These endosperm mutants have been invaluable tools in starch and storage protein studies (Shannon and Garwood 1984). Therefore, mutant endosperm cultures could provide a new experimental system. However, since endosperm mutants are expressed specifically in only endosperm and pollen, there is no reason to expect the mutant phenotypes to be expressed in in vitro cultures.

In this study, we tested two necessary conditions for the development of mutant maize endosperm cultures for use in genetic and physiological studies. First, we demonstrated the feasibility of growing mutant endosperm in culture by utilizing backcross conversions of the maize genes *waxy* (*wx*) and *amylose-extender* (*ae*) to inbred line A636 for the establishment of vigorously growing mutant cultures. Second, the phenotypic expression of *ae* and *wx* in culture was tested. Starches from normal and mutant kernels were compared to starch that was formed in the same genotypes in culture. Comparisons were made based on starch granule morphology and composition.

Materials and methods

Genetic material

The inbred line A636 was used as a source of material. Plants were either 'normal' or isogenic (5 backcross generations) for the mutants *amylose-extender* (*ae*) or *waxy* (*wx*). Plants were self-pollinated. Endosperm tissue was taken from kernels 10 to 12 days after pollination for culture initiation. Immature ears were harvested at 18 days after pollination, frozen on dry ice and freeze dried.

Endosperm culture-initiation and maintenance

Ears were sterilized in 70% ethanol for 5 min and then in 0.525% sodium hypochlorite for 5 min and rinsed twice with sterile water before excision. The endosperm was removed by slicing off the pericarp and aleurone layers at the top of the kernel and removing the endosperm tissue with a microspatula (Shannon 1982). Four explants were placed on a 5 ml slant of modified Linsmair & Skoog's medium (LS) (1965) containing 0.4 mg/l thiamine, 2 g/l asparagine, and 3% sucrose (Shannon and Liu 1977). The cultures were kept in a dark growth chamber at 28 °C (LaRue 1949).

Twenty-one days after initiation the explants were placed on 50 ml of solid medium in 250 ml flasks. Six explants were placed in each flask. After 14 to 21 days the callus tissue was placed in 250 ml Erlenmeyer flasks containing 80 ml liquid medium. The cultures were subcultured on a 14-day schedule with 5 ml of tissue per 80 ml of medium added to each flask (Shannon 1982).

Mother cultures were maintained for each of the mutants and normal endosperm. To optimize starch production, cultures were transferred to LS medium containing 2% sucrose. Nine days after subculture these cultures were supplemented with a sterile sucrose solution (6 g in 10 ml distilled water) (Chu and Shannon 1975). The cultures were harvested 16 days after the sucrose supplementation. Experiments were performed 9 and 15 months after culture initiation.

Starch isolation

Starch was isolated cultures, mature kernels and kernels harvested 18 days after pollination following the procedures of Boyer et al. (1976c). Purified starch was dried, weighed and used directly. Starch production was calculated on a percent culture dry weight basis.

Fractionation of amylose and amylopectin

Twenty milligrams of starch was dissolved in 2 ml of 95% DMSO and precipitated with 3 volumes of 75% MeOH and 1% KCl. The precipitate was dissolved 2 ml of 0.01 M NaOH and 0.02% Na azide solution and 1 ml of this solution loaded on to a Sepharose 2B-Cl gel filtration column (1.5 × 50 cm). Fractions (1.5 ml) were collected and fractions assayed with iodine reagent (Boyer et al. 1976b). Peaks were pooled into 2 samples (Yeh et al. 1981), and the quantity of carbohydrate present in each peak was measured by the phenol sulfuric acid procedure (Hodge and Hofreiter 1972).

Starch granule morphology

Starch granule structure was observed in isolated starch samples using light microscopy. The organization of endosperm and callus tissue was also determined. Samples were fixed with 3% glutaraldehyde in 0.15 M sodium cacodylate buffer at a pH of 7.1 for 1.5 h. An equal amount of 1% osmium tetroxide in 0.15 M sodium cacodylate buffer was added to the samples and they were placed in an ice bath. The glutaraldehyde-osmium mixture was decanted off after 30 min and 1% osmium in the above buffer was added to the tissue for 1 hr. The tissue was rinsed and sequentially dehydrated with graded acetones. The embedding of the tissue was done by adding a graded series of epoxy/acetone solutions (Spurr 1969). Thick sections were stained using toluidine blue.

Results and discussion

Genetic control of in vitro endosperm growth

The ability to produce actively growing callus cultures of many plants often has been shown to be line (or variety) dependent. Shannon and Batey (1973) suggested that the growth of maize endosperm in culture was under genetic control and identified inbred line A636 as being superior for this purpose. If this is true, utilization of the backcross procedure with inbred A636 as the recurrent parent should recover the ability to produce vigorous growing cultures. In this study, the mutant alleles of the *ae* and *wx* genes were transferred

from inbred Oh43, a very poor line for establishment of endosperm cultures (Shannon and Batey 1973). The backcross lines used here resulted from five backcross generations and therefore, would be over 98% converted A636 or near isogenic. All three endosperm types, normal, *ae* and *wx*, produced vigorously growing cultures. These results support the genetic basis for establishment and growth of maize endosperm in vitro. Therefore, any mutant endosperm could be grown in vitro after incorporation into the A636 inbred by backcrossing.

The tissue cultures derived from *ae*, *wx* and normal endosperm exhibited some differences. Most of the *ae* cultures grew fairly rapidly and produced small clumps of yellow callus tissue. The *wx* and the normal cultures grew much more slowly and produced large clumps of white callus tissue. Shannon and Liu (1977) and Shimamato et al. (1983) also found similar size and morphological differences between the cell lines of normal endosperm that they established, so these differences are probably not a result of the presence of the mutant characteristics, but of some other origin.

The percentage of starch per g dry weight produced in endosperm cultures of normal, *wx*, and *ae* was highly variable. Samples with similar fresh weights and dry weights did not necessarily produce similar amounts of starch (3 to 23% starch/g dry wt). However, samples produced from a given culture line produced similar amounts of starch (coefficient of variation = 10–20%).

The percentage of amylose

Starches from normal, *ae* and *wx* callus and endosperm tissue were examined by Sepharose 2B-CL gel filtration. Typical profiles of starch fractionated by this procedure are shown in Fig. 1. Amylopectin elutes first (column fractions 11–20) and amylose elutes second (column fractions 21 to 40). The amylopectin peaks had maximum absorbances of the iodine-starch complexes at 530 to 550 nm while the amylose peaks had a maximum iodine-starch absorbances of 620 to 630 nm. Elution profiles of *ae* callus and endosperm starches showed the same peaks as normal starches (not shown). Only one peak (amylopectin) was found in gel filtration elution profiles of starches from *wx* callus tissue and endosperm (not shown).

The percent amylose of starch samples was estimated from the gel filtration experiments. Amylose content in starches from *wx* endosperm cultures and *wx* kernels was 0 (Table 1). Starches in 9 month old cultures of *ae* endosperm tissue contained from 30.2% to 47.6% amylose with a mean value of 39.9%, while the percent amylose in normal callus tissue ranged from 24.2% to 38.6% with a mean value of 28.2% (Table 1). There was some overlap between the *ae* samples that contained less amylose and the normal samples that were higher in amylose, but the mean amylose content in *ae* callus tissue was significantly higher than that of starch from normal callus tissue. Starch from normal endosperm tissue from mature kernels contained 31.7% amylose. The percent amylose in normal kernel tissue was not significantly different than that of normal callus tissue. The amylose percentage of *ae* endosperm tissue from mature kernels was 67.7%. This was signifi-

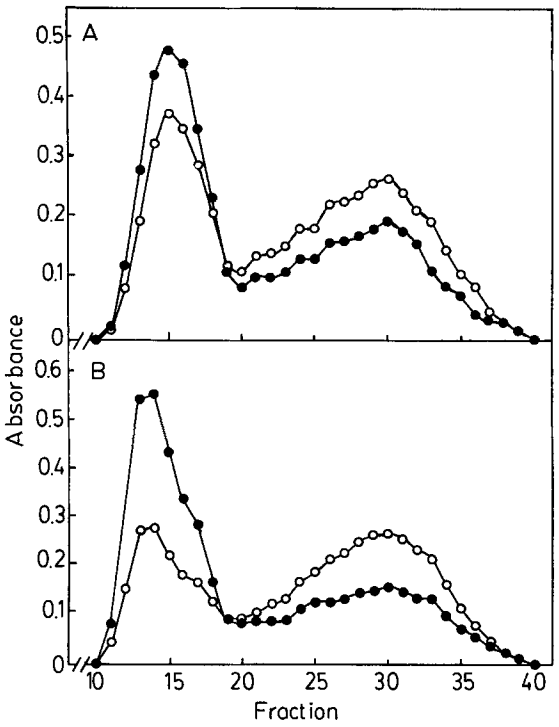


Fig. 1 A, B. Gel filtration elution profiles of starches from normal endosperm and cultures, –●– A_{540}; –○– A_{660}. **A** Starch from mature endosperm. **B** Starch from 9 month old endosperm cultures

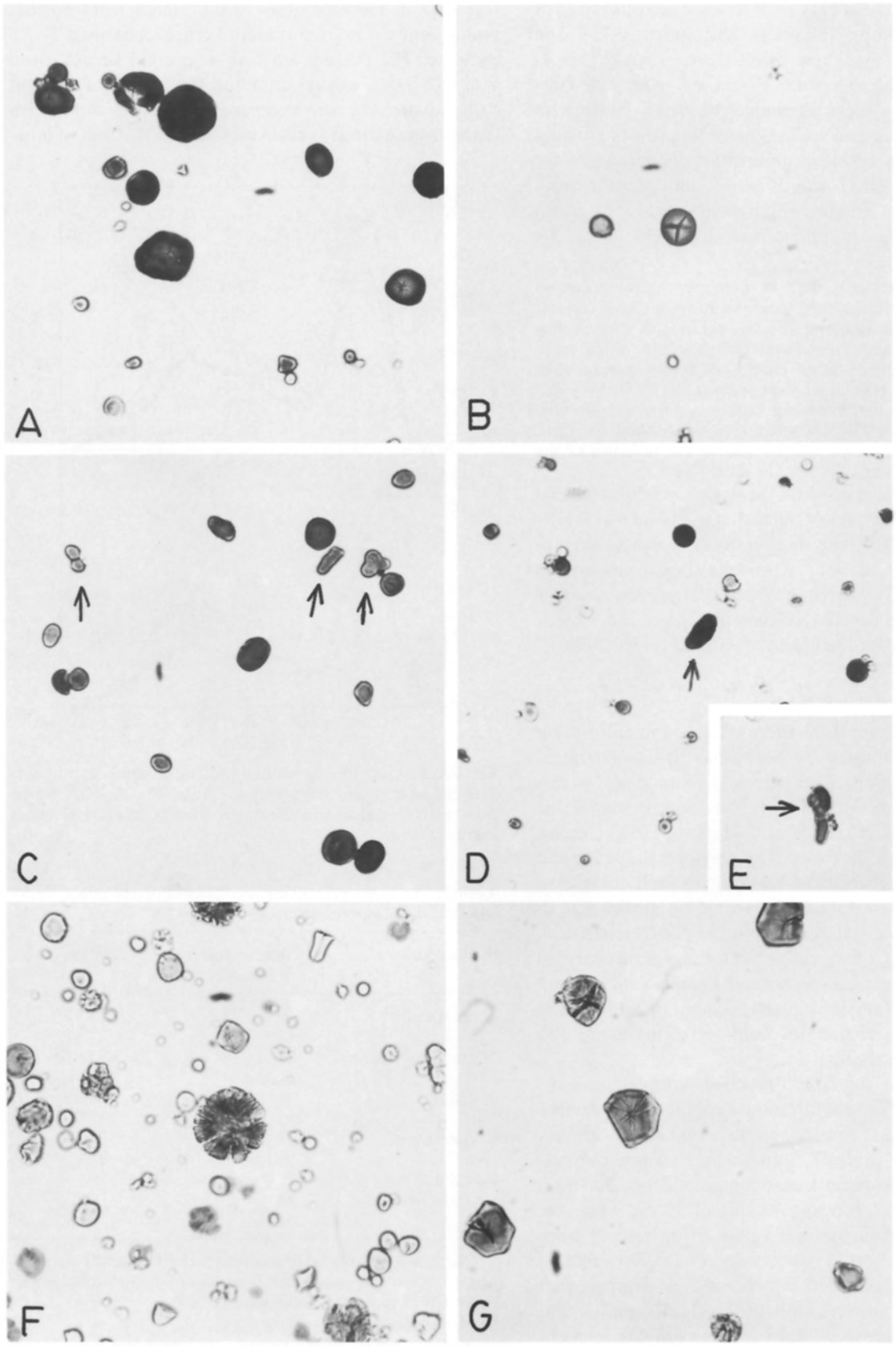
Table 1. Amylose percentage of starches from intact endosperm and endosperm cultures

Starch source	Time in culture or maturity[a]	% amylose[b]
Normal callus	9	28.2c[c]
	15	22.5d
Normal endosperm	18	17.9d
	mature	31.7c
ae callus	9	39.9b
	15	29.2c
ae endosperm	18	31.1c
	mature	67.7a
wx callus	9	0
wx endosperm	18	0e
	mature	0e

[a] Starches were isolated from callus 9 and 15 months after culture initiation and endosperm from mature kernels and kernels harvested 18 days after pollination

[b] Mean values from 4 to 20 samples

[c] Means followed by a common letter were not significantly different at using the Waller Duncan k-ratio *t*-test (k = 100)


A
B
C
D
E
F
G

cantly higher than the percent amylose of *ae* callus tissue and also of normal endosperm and normal callus tissue.

The differences in the percentage of amylose in mature *ae* endosperm and *ae* callus tissue may be due to the stage of development of the amyloplasts. Boyer et al. (1976c) observed that in the intact kernels, the level of amylose increases as the kernel matures, with the largest accumulation between 18 and 36 days after pollination, while in callus cultures, the cells are stimulated to increase starch production 9 days after subculture and are harvested 16 days later. Possibly, the percent amylose would continue to rise after 16 days following sucrose addition, however, Chu and Shannon (1975) reported that maximum starch accumulation occurred by 16 days after supplementation and then declined. Although, the percent amylose in starches from 9 month-old normal endosperm cultures was fairly similar to that of starch from normal endosperm tissue, after 15 months in culture, the amylose content of normal starches was also lower than in starch from mature kernels (Table 1). The amylose content of starch of *ae* cultures 15 months after culture initiation was also lower (Table 1). In both cases, the amylose content of the starch more closely approximated the amylose contents of starches from 18-day-old endosperm of the same genotype. It may be that the 9 month-old normal cultures reached a higher level of maturity than the *ae* cultures, or that because the increase in the percent amylose is more dramatic in the *ae* tissue than in the normal tissue, differences for starches from normal endosperm and cultures were not observed at 9 months. Approximately a 14% increase in amylose content of starches from normal endosperm tissue and a 26% increase in *ae* endosperm starch occurred from 18 days after pollination to maturity (Table 1). By 15 months, the cultures of both *ae* and normal have further lost the ability to produce mature amyloplasts and the difference between starches produced in cell culture and mature endosperm are magnified. In spite of the difference between cultures and intact endosperm, genotype differences were maintained in culture up to 15 months after initiation. That is to say, *ae* cultures produce "higher" amylose starches than normal while *wx* culture produce only amylopectin. Thus, endosperm cultures can provide future models for endosperm gene expression. The variation between samples of the same genotype, however, needs to be overcome.

Light micrographs of isolated starch granules

Mutant effects can also be examined at the level of starch granule morphology. Starch granules from normal callus tissue were similar in shape to those found in mature normal endosperm tissue except there were more small starch granules present in the normal callus samples (Fig. 2). Starch granules from both normal callus and normal endosperm were spherical to polygonal in shape. The presence of smaller starch granules in the normal callus preparations may be due to a younger physiological age of some of the normal callus cells, although the percent amylose of starches from these cultures was not significantly different than that in starch from normal mature endosperm cells.

In starch samples isolated from *ae* callus tissue, the starch granules were highly varied in size (Fig. 2D). Starch granules isolated from mature *ae* endosperm tissue were larger and more uniform in size (Fig. 2C) than in *ae* callus, but were still smaller than normal or *wx* starch granules. In addition, many irregular starch granules were observed in mature *ae* endosperm tissue (Fig. 2C) while only a few iregular starch granules were observed in samples from *ae* callus tissue (Fig. 2D). The small number of irregular starch granules in *ae* callus samples is possibly related to the significantly lower percentage of amylose in starches from *ae* callus tissue as compared to the mature *ae* endosperm tissue. This is supported by the observations of Boyer et al. (1977) which showed that increases in the percent amylose and abnormally shaped starch granules in developing maize endosperm tissue were highly correlated. Abnormal granules seem to result when the amylose content is above 30% (Boyer et al. 1977).

The starch granules in *wx* callus samples (Fig. 2G) varied in size like those found in normal and *ae* callus samples. Although the starch granules from *wx* callus tissue were primarily spherical in shape, some of the larger starch granules had jagged edges. Starch granules from mature *wx* endosperm tissue were more angular in shape but also contained starch granules with jagged edges (Fig. 2F). The jagged edged starch granules were unexpected and have not previously been reported in *wx* starch samples. However, the appearance of these granules in starch from intact endosperm and cultures further demonstrates the expression of the *wx* endosperm genotype in vitro.

Light micrographs of callus cells

Light micrographs of callus tissue from normal, *ae*, and *wx* endosperm cultures displayed a wide variety of cells. Neighboring cells many times contained extremely different structures. The cell contents ranged from largely vacuolar to very compact.

Boyer et al. (1976a, 1977) observed a gradient of starch accumulation in maize endosperm tissue from the central crown calls to the basal portion of the endosperm and also from the central crown cells outward. A similar pattern of starch accumulation in cultures was observed in this study. Starch accumulation in endosperm cultures appears to begin in 'pockets' of cells throughout the tissue (Fig. 3A, B). The lack of synchrony between callus cells during starch accumulation may be due to the age differences among cells.

Fig. 2 A–G. Light micrographs of starch granules isolated from intact endosperm and 9-month-old endosperm cultures. Starch granules were stained with iodine for contrast. **A** Normal endosperm; **B** Normal endosperm cultures; **C** *ae* endosperm (arrow indicate abnormal granules); **D, E** *ae* endosperm cultures (arrows indicate abnormal granules); **F** *wx* endosperm; **G** *wx* endosperm cultures. All magnifications are ×400

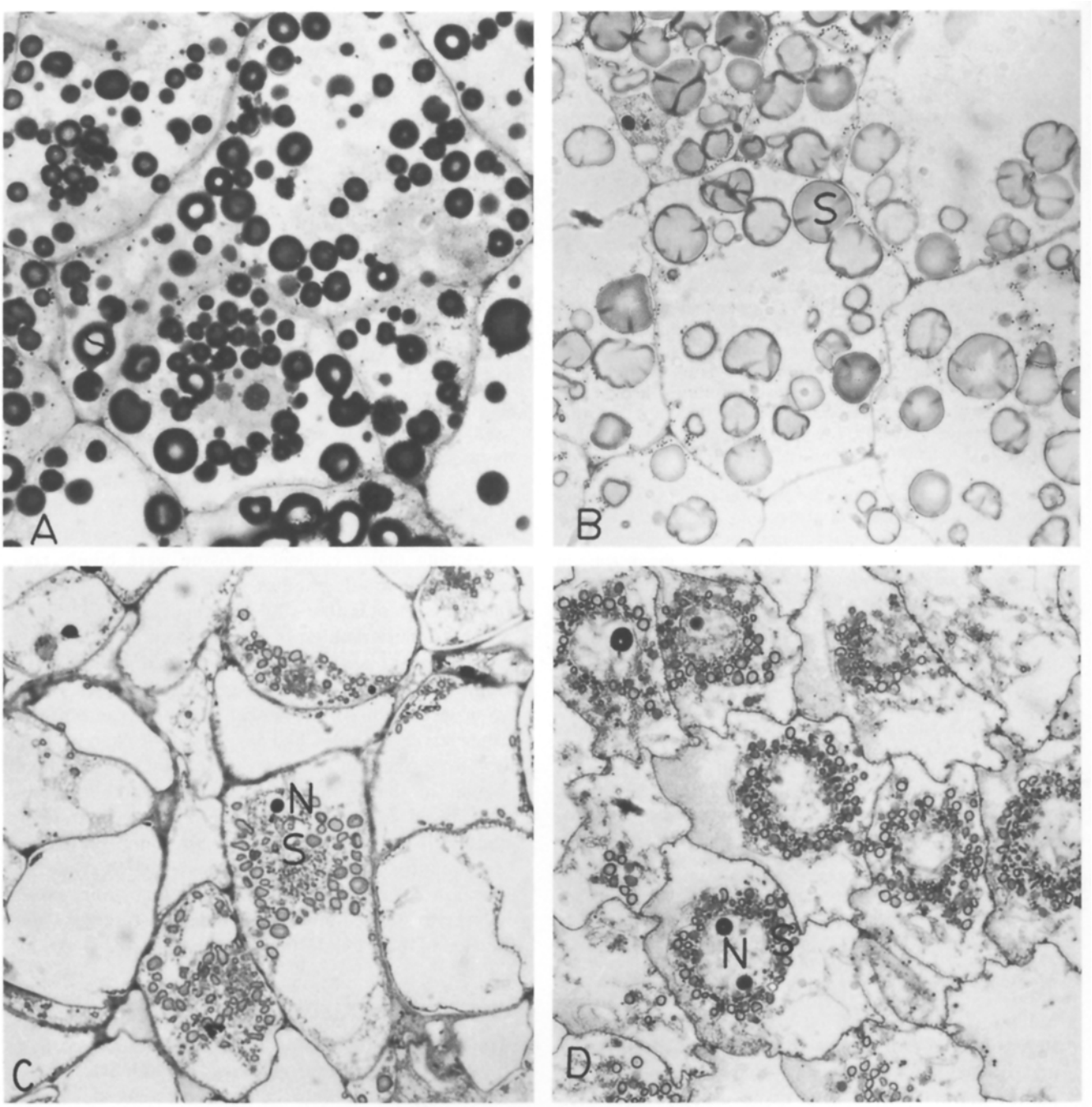


Fig. 3 A–D. Light micrographs of sections of intact endosperm and endosperm cultures: S = Starch granule; N = nucleus. **A, B** Pocket of starch granules accumulating in normal (**A**) or *wx* (**B**) callus cells (×400); **C** Starch granules accumulating around the nuclei in *wx* callus cells (×250); **D** Starch granules accumulating around the nucleus in 18-day old normal endosperm tissue (×400)

Many of the cells may not be at the same stage of development, some cells show signs of scenecence, while others are actively dividing. The inability to stimulate starch accumulation in the majority of cells at the same time may be one of the major factors leading to low levels of starch production in vitro. In cells accumulating starch, starch granules appear to accumulate either around the nucleus or along the periphery of the cell in endosperm callus cultures (Fig. 3C). The accumulation of starch in intact kernel tissue appears to be analogus to this (Fig. 3D). Boyer et al. (1977) reported similar starch granule formation pat-

terns in developing maize endosperm. A detailed study of endosperm callus cell ultrastructure is in preparation (F. Felker, personal communication).

Conclusions

Specific effects of the endosperm mutants *ae* and *wx* can be seen at several levels of organization. First, both *ae* and *wx* alter the accumulation of amylose in the starch granule. Second, genotype related difference in starch granule morphology were observed. By examining these properties of starches from normal, *ae* and *wx* endosperm cultures and comparison with starches from intact endosperm of the same genotype, we have demonstrated the phenotypic expression of *ae* and *wx* in vitro. We conclude that endosperm specific gene expression occurs in cultures of maize endosperm, but variation within a genotype occurred. Therefore, cultured endosperm could be used in future studies of maize endosperm gene expression and metabolism.

Note added in proof

After preparing this manuscript, we have established actively growing cultures of *opaque-2* (*o2*) and *sugary* (*su*) endosperms using A636 backcross conversions. Characterization of the *sugary* cultures is now in progress. Small supplies of normal A636 and the A636 backcross conversions for the endosperm genes *ae, o2, su* and *wx* are available on request from C.B.

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